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BIOSYNTHESIS OF DEUTERATED LIPIDS FOR LUBRICANT APPLICATIONS USING THE GREEN ALGA, *SCENEDESMUS OBLIQUUS*

Report No 91-87-K

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ABSTRACT

The green alga, *Scenedesmus obliquus*, was grown photosynthetically in concentrated heavy water (99.6%) medium in closed batch culture. Cell components were biosynthesized using only the inorganic precursors supplied during growth. Algal biomass yields averaged 0.84 g dry wt.L^{-1} which was about 56% of the biomass yields obtained from H_2O medium. Growth rates were 3 to 7 times higher on H_2O medium as compared to D_2O medium. Under routine culture conditions cell lipid content averaged 10% of cell dry weight in both media. The perdeuterated fatty (carboxylic) acids derived from the lipid component of cells cultured in D_2O were palmitic acid (16:0; 58.9%), oleic acid (18:1; 24.0%) and cis,cis-11,14-eicosadienoic acid (20:2; 17.1%). A new method, involving the use of calcium oxide to facilitate flocculation and settling of algal cells followed by direct saponification of the concentrated cell mass, was an effective and promising alternative to centrifugation, lipid extraction and saponification for the recovery of fatty acid products.

job	file	date	report no.
740634-501-002	831.36	April 18, 1991	91-87-K

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EXECUTIVE SUMMARY

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Chemical Research Department

The biosynthesis of deuterated lipids was investigated as a potential alternative to chemical substitution for the production of fully deuterated starting materials that could be used in synthetic lubricants. The photosynthetic green alga, *Scenedesmus obliquus*, was used in this study since it can produce neutral lipids (triacylglycerols) in relative abundance and it is able to grow in concentrated (>99.6%) heavy water medium. Autotrophic growth of this alga in a closed heavy water culture system ensures the incorporation of deuterium (as opposed to hydrogen) into cell material; only inorganic precursors and the energy provided by light are required for biosynthesis.

A prolonged adaptation period, in excess of three months, was required to establish the growth of *S. obliquus* in concentrated heavy water medium. During this adaptation period, each step-wise increase in D_2O concentration resulted in reductions in growth rates and culture yields. An exceptionally long lag growth phase (time that precedes commencement of growth in new medium), in excess of one month, occurred when heavy water concentration was increased from 97.5% to 99.3%. Once established in concentrated heavy water medium, however, good growth of *S. obliquus* was obtained and these cultures have now been maintained for over three years.

Compared to H_2O cultures, the lag growth phase of D_2O cultures is longer (eg, 4 days versus 1 day), growth rates are 3 to 4 times slower, and biomass yields are lower (0.84 g/L versus 1.5 g/L). Lipid yields from cultures harvested immediately following exponential growth phase represented about 10% of biomass dry weight for both D_2O and H_2O cultures. The grow out period for these cultures was about 18 and 10 days, respectively. Much higher biomass and lipid yields could be expected under culture conditions that have been optimized for growth (ie, not light limited) and for subsequent lipid production.

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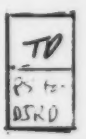
S. obliquus tolerated a wide range of copper concentrations (0.22 to 18.3 mg/L) indicating the potential for enhancing lipid production by screening for copper tolerant strains.

Yields of end product fatty acids averaged 3% to 5% of biomass dry weight. The predominant fatty acids from algae grown in H_2O medium, and the only identified fatty acids from cells grown in D_2O medium, were palmitic (16:0), oleic (18:1) and 11,14-eicosadienoic acid (20:2). The relative proportions of these fatty acids from algae grown in D_2O medium were 59%, 24% and 17%, respectively. These fatty acids were more than 95% deuterated. Algae grown in H_2O medium contained up to six additional fatty acids, with stearic acid (18:0) a component of all H_2O samples. The proportion of saturated to unsaturated fatty acids was similar for algae grown in H_2O and D_2O media.

Fatty acid yields were compared for algal material harvested by centrifugation or chemical flocculation (using calcium oxide) which was either directly saponified or saponified following methanol-chloroform extraction of lipids. Mean yields differed by a factor of two among the four treatments although the differences were not statistically significant. The use of CaO to facilitate flocculation and settling of algal cells followed by direct saponification of the concentrated cell mass, was an effective and promising alternative to centrifugation, lipid extraction and saponification for the recovery of fatty acid products. However, further testing of this new procedure is recommended.

Algae grown photosynthetically are capable of producing a wide range of perdeuterated organic compounds. Two important advantages of biosynthesis over deuteration via chemical substitution include ease of production and the ability to deuterate labile organic compounds.



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To: Dr. D. Mills
Director of Research

BIOSYNTHESIS OF DEUTERATED LIPIDS FOR LUBRICANT APPLICATIONS USING THE GREEN ALGA, *SCENEDESMUS OBLIQUUS*

INTRODUCTION

Deuterated synthetic lubricants, which have greatly enhanced thermal and oxidative stability and hence a longer useful lifetime than their proteated counterparts, were identified as a potential non-nuclear market for heavy water by Robins (1984a), in a review undertaken at the request of New Business Ventures. This was followed by the establishment of a research and development initiative, funded by the Isotope Sales and Service Program, with the goals of investigating the properties and performance of deuterated lubricants and identifying potential means for producing these materials. In addition to lubricants, deuterated organic materials may have other applications where deuteration provides an advantage over their proteated counterparts (Robins 1984a).

The enhanced resistance to oxidation of deuterated compounds results from the lower reactivity of the carbon-deuterium bond as compared to carbon-hydrogen bond (Schindler and Wolock 1982, Campana 1983, Mullins et al. 1988). Reactive hydrogen bonds leading to the degradation of the lubricant, pentaerythritol tetrahexanoate, were located on the hexanoate portion of the polyol ester. Furthermore, a high deuterium content was required to obtain large improvements in oxidation resistance (Schindler and Wolock 1982). Methods were therefore sought for a cost-effective means of producing perdeuterated alkanoate starting materials for lubricant applications.

Chemical procedures can be used to replace the hydrogen content of organic compounds with deuterium. This is a random process that results in deuterium-hydrogen exchange, with the extent of exchange depending on exposure time and inherent stability of the starting material (Campana 1983, Tulloch 1979). Current costs for chemically substituted perdeuterated organic materials have, however, greatly restricted the use of these materials and have focused attention on other, more economical means of producing these materials (Robins 1984a, b, Schindler and Wolock 1982).

The possibility of biosynthesizing perdeuterated fatty (carboxylic) acids for use as starting materials in lubricants was considered as a possible alternative to chemical synthesis (Campana 1983, Niehof et al. 1986, Dunstall 1985). Criteria used in the selection of candidate organisms for in-house laboratory evaluation of deuterated lipid production included the ability of the organisms to grow autotrophically (ie, photosynthetically) using only inorganic precursors during biosynthesis, rate of biomass production, cell size (for harvesting purposes), yield and extractability of suitable lipids (ie, acylglycerols), and requirements pertaining to the ability of the organisms to grow in concentrated heavy water (> 99.6%).

The green alga, *Scenedesmus obliquus* (Chlorophyceae), met the established criteria and was selected for future laboratory testing to evaluate the efficacy of the biosynthetic option (Dunstall 1985). Autotrophic algae use the energy derived from light during biosynthesis and incorporate exogenous deuterium from the growth medium (ie, heavy water) into newly synthesized organic compounds. The neutral lipids (acylglycerols) produced by *S. obliquus*, and other species of green algae, are easily extracted using non-polar solvents (Lien and Spencer 1984). The 16 and 18 carbon fatty (carboxylic) acids that are predominant in these algae can be extracted and cleaved to form 5 to 11 carbon deuterated alkanoic acids. These acids would then be used as starting materials for the production of synthetic lubricants (e.g. pentaerythritol perdeuteroalkanoates).

The objectives of this project, therefore, were to establish capability for the biosynthesis of fully deuterated lipids from algal culture, develop the methods for the recovery of fatty acid end products and to provide a preliminary evaluation of this production method. Procedures for harvesting microalgae and for extracting fatty acids from algal cells were therefore examined. Concurrent with this project, comparable studies were being conducted by Martek Corporation of Columbia, Maryland (Kyle et al. 1987), and by the US Naval Research Laboratory (Niehof et al. 1986, 1987, Ross et al. 1986).

MATERIALS AND METHODS

Scenedesmus obliquus (strain 393) was obtained from the Culture Collection of Algae, University of Texas. The alga was established in three media: Beijerinck's (Nichols 1975), NRL - high iron and NRL - low iron (modified after Niehof et al. 1986; Appendix I). Growth in all three media was initially slow but gradually increased following successive sub-culturing into fresh media. These cultures were maintained at approximately 20°C. Culture growth (cell density) was followed by measuring the light absorbance of 4 mL samples (neat or diluted) at 685 nm.

Adaptation and Growth of *S. obliquus* in Heavy Water Medium

The NRL high iron medium was initially used to adapt the alga to step-wise increases in heavy water, at concentrations of 33.3%, 62.2%, 73.7%, 85.6%, 93.4%, 97.5% and 99.3, and 99.6% (concentrations after inoculation). Increases in concentration above 93.4% were also carried out using Beijerinck's medium. Algal growth was obtained at each successive D_2O concentration before attempting to adapt to a higher concentration. A relatively large inoculum, generally 20 mL, was transferred into 100 mL of fresh medium (ie, a 17% inoculum) poised at successively higher D_2O concentrations. Triplicate cultures were used for each D_2O concentration.

Closed batch cultures were used to minimize both water loss through evaporation and the exchange of H_2O in water vapour with medium D_2O. Cultures were established in round-bottom Pyrex flasks containing approximately 5-L Beijerinck's medium prepared from concentrated heavy water (>99.6%). Hydrogen introduced into heavy water medium by the addition of nutrient salts was insignificant. In each trial, two culture vessels were inoculated with adapted starter cultures that were in exponential growth phase; inoculum size = 3 to 5% of the total culture volume.

Culture flasks were maintained at approximately 25°C (range = 20 to 28°C) under constant illumination (Figure 1). Fluorescent light banks (4 X 40 W) provided one side of each flask with approximately 120 uE/s/m^2, while the opposite side of the flasks was illuminated with two 40 W fluorescent bulbs. Air enriched with 5% carbon dioxide was supplied to the bottom of the culture vessels at a rate of approximately 4 L/h/flask. The air lift system provided the carbon source for cell growth and, in conjunction with magnetic stirring rods, maintained the cells in suspension thereby ensuring light availability. Prior to delivery to the flasks the CO_2-air mixture was passed through a heavy water wash (pH 8.5). Previous experience with similar culture systems indicated that the change in culture volume during incubation was generally less than 1% (Dunstall 1984).

Processing of Cell Material

A combination of procedures were used in an attempt to expedite downstream processing. Two methods, centrifugation and sedimentation aided by chemical flocculation, were used to harvest cells. Concentrated cell material, obtained using each of these two methods were then subjected to two procedures for obtaining fatty acid end products: (i) extraction of lipids and subsequent saponification, and (ii) direct saponification of concentrated algal material.

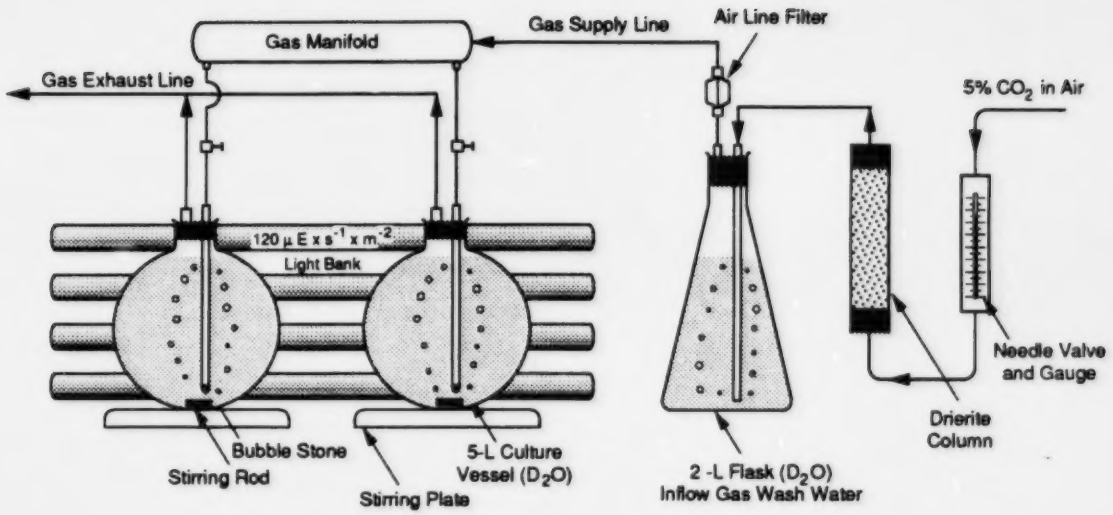

FIGURE 1

ALGAL CULTURE APPARATUS

Cell Harvesting

Algal cells were concentrated by centrifugation (10 min; 3,500 g) or, alternatively, by adding the flocculating agent, calcium oxide, to culture medium (0.4 g/L culture medium), thereby facilitating the sedimentation of cells. Calcium oxide was added to a small volume of culture medium in the bottom of a settling cylinder and swirled to dissolve the flocculating agent. The remainder of the culture was then added to the cylinder, stirred well, and left for approximately 0.5 h to settle. (The precipitate that forms at a pH of about 11.5 attaches to the algal cells thereby facilitating the settling.) Once the cells had settled the clear supernatant was removed using a siphon. Prior to harvesting, the dry weight of the algal cells was determined by filtering 5 or 10 mL of culture onto predried and preweighed Whatman GFC filters (1.2 µm) and oven drying at 100°C to constant weight.

Lipid Extraction

The algal slurry that remained following settling of the flocculated algal cells was acidified to pH 6 to 7 and centrifuged. The collected pellet was resuspended in a small volume of water forming a cell paste (50-100 mg dry wt./mL water; H_2O or D_2O depending on initial culture medium). Similarly, a cell paste was made from the pellets resulting from direct harvesting of algal cells by centrifugation. These cell pastes were heated for 1 to 2 min in a boiling water bath to rupture the cell walls, followed by cooling in a cold water bath (Graff et al. 1970).

Lipids were extracted using a methanol-chloroform mixture following the methods of Blight and Dyer (1959) as modified by Kates (1972) and Dubinsky and Aaronson (1979). Methanol and chloroform were added to the cell debris in a volume ratio of 2:1:0.8 (methanol:chloroform:water) and the mixture was shaken vigorously. Additional chloroform and water were added, shaking vigorously between each step, to give a final ratio of 2:2:1.8. The mixture was centrifuged and the lipid-containing chloroform phase recovered. The residue was rehydrated (H_2O or D_2O depending on culture medium), acidified with HCl, chloroform was added, and the mixture was centrifuged. The supernatant was combined with the methanol-water fraction from the first extraction (ratio = 2:2:1.8). The chloroform phase from this acid-treated sample was recovered and combined with the chloroform fraction from the first extraction. (Note: Lipids from the January 22 and February 25 cultures were extracted in the same manner except that the residues obtained from the first extractions were not acidified.) Lipid weight was determined following evaporation of the chloroform which was aided by gentle heating (35-40°C), occasional additions of benzene and the passage of dry nitrogen over the surface of the liquid. It should be noted that all substances having an aliphatic hydrocarbon chain (ie: fatty acyl acids) and substances soluble in

these materials are included in the recovered lipid fraction (Lehninger 1970, Ratledge 1982).

Saponification

Lipids extracts (ie, acylglycerols) were hydrolyzed to glycerol and component fatty acids using the method of Farag et al. (1983). Concentrated lipid was dissolved in 50 mL ethanol, adjusted to pH 12 with KOH, and refluxed for 2 h. Subsequently, water (100 mL) was added to the sample and the pH was adjusted to 7 using HCL. The liberated fatty acids were extracted with hexane (100 mL). Two additional hexane extractions were performed on the aqueous phase. The combined hexane extract was dried over anhydrous sodium sulphate, passed through Whatman #1 filter paper and the filtrate evaporated in Rotovap evaporator. Final evaporation was carried out in preweighed flasks to obtain fatty acid dry weights. Fatty acids were redissolved in a small volume of hexane for storage.

The method for the direct saponification of concentrated algal material, a variation of the procedure of Farag et al. (1983), was developed by Dr. H. Azizian (Materials Chemistry Section). One problem was encountered with samples in which CaO had been used as a flocculating agent. It was difficult, because of the presence of CaO in the cell debris, to readjust these samples to pH 7 following the reflux step. This problem was resolved by separating the cells (and CaO) from the ethanol-water fraction, washing this debris twice with additional ethanol, and then adjusting the pH of the combined ethanol-water fractions to 7.0. Following the ethanol wash, the cell debris-CaO fraction was also washed with 20 mL hexane which was added to the combined hexane fractions described above. This modified method was used on the concentrated algal material obtained in the final 2 experiments, June 18 and July 10, by both centrifugation and CaO flocculation.

Fatty Acids Analysis

Mixtures of fatty acids obtained following saponification were analyzed by the Analytical Services Section (Chemical Research Dept.). In total, five fatty acid samples derived from normal water culture and three samples from heavy water culture were analyzed. Samples were prepared by converting the fatty acids to their corresponding methyl esters by mixing 1 mL toluene and 1 mL methanolic-base derivatizing reagent with 10 to 30 mg of sample in reacti-vials. The solution was heated to 80°C for 20 min, cooled, and then 3 mL of water and 3 mL dichloromethane was added and the solution was extracted for 2 min. Dichloromethane extracts (2 µL) were analyzed by capillary gas chromatography-electron impact mass spectrometry by comparison to reference standards. Instrument set points are detailed in Massey (1988).

Copper Tolerance

It was initially intended to examine the influence of copper concentration and nitrogen source (NO_3 or NH_4) and concentration on cell lipid content with the aim of maximizing the production of lipid. However, only preliminary trials designed to evaluate copper tolerance by *S. obliquus*, a prelude to the selection of a cell lines with enhanced lipid content, were conducted.

Copper concentrations that ranged from 0.220 to 220 mg/L were established in H_2O Beijerinck's medium. The copper concentration of standard Beijerinck's medium is 0.385 mg/L. Copper was added as $CuSO_4.5H_2O$. Cells in exponential growth phase were inoculated into 100 mL of medium poised at different copper concentrations and growth was followed by measuring light absorbance at 685 nm.

RESULTS

Adaptation and Growth of *S. obliquus* in Heavy Water Medium

The lag growth phase in batch cultures characterizes the period required for cells to adapt to new (stressed) conditions, and precedes the proliferation of cells. This lag phase is followed by a period of exponential cell growth in which cell biomass doubles within constant time intervals. The rate of growth is also affected by the suitability of culture conditions.

The initial transfer of algal cells from H2O medium to 33.3% D_2O medium resulted in no noticeable change in growth. In subsequent transfers to medium containing 62.2%, 73.7% and 85.6% heavy water, the lag period remained largely unchanged, lasting from 1 to 3 days (Figure 2). Cultures having higher initial cell densities were characterized by somewhat shorter lag periods. Upon transfer to 93.4% and then 97.5% heavy water the lag period increased slightly, to approximately one week. However, beyond 33.3% D_2O, the growth rates (and biomass yields) declined upon each transfer to successively higher heavy water concentrations, up to 97.5% D_2O (Figure 3).

In NRL medium containing 93.4% heavy water the cells had acquired a slightly chlorotic appearance and thus the transfer to 97.5% D_2O medium was carried out using both NRL and Beijerinck's media. The ensuing transfer from 97.5% to 99.3% heavy water resulted in a prolonged adaptation period, in both media, which lasted 30 to 39 days. The lag phase in Beijerinck's medium tended to be slightly longer. These prolonged lag periods following initial transfer to 99.3% medium prompted a number of additional attempts, using the 97.5% D_2O cultures as inocula, to establish growth. It was generally observed that the lag period could be halved by using older cultures for the inoculum and by increasing the inoculum volume. Once the cells had adapted to 99.3% D_2O, growth rates and

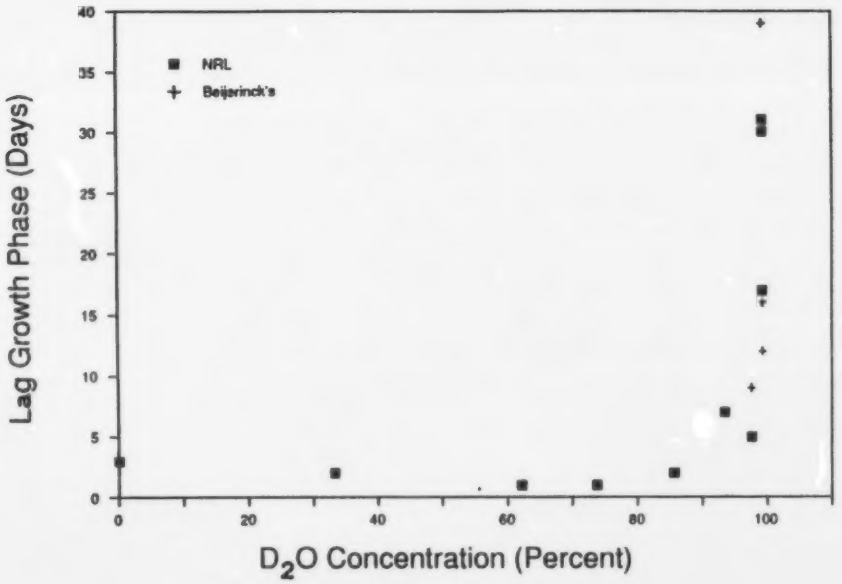

FIGURE 2

LAG GROWTH PHASE FOR CULTURES OF *S. OBLIQUUS* DURING ADAPTATION TO CONCENTRATED HEAVY WATER MEDIUM

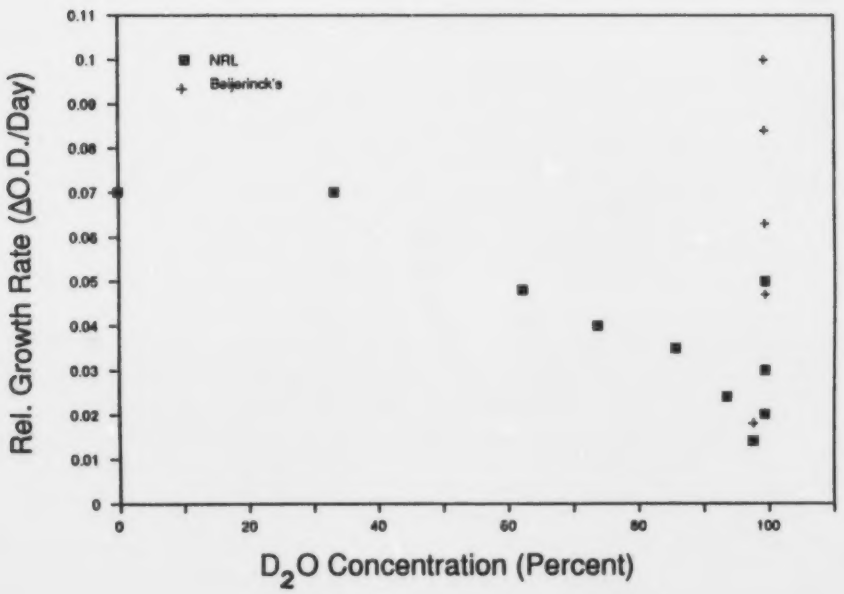

FIGURE 3

RELATIVE GROWTH RATES FOR CULTURES OF *S. OBLIQUUS* DURING ADAPTATION TO CONCENTRATED HEAVY WATER MEDIUM

biomass levels were up to three times greater on Beijerinck's medium (eg, Figure 4). Cells grown on Beijerinck's medium were also healthier looking compared to NRL cultures which remained somewhat chlorotic. It had also become apparent by this time that growth of the alga in Beijerinck's (H_2O) medium was much better than growth in either NRL high iron or NRL low iron media. Further use of NRL medium was therefore discontinued in favour of Beijerinck's medium.

It took just over three months to establish *S. obliquus* in concentrated (>99.6%) heavy water medium. With subsequent subculturing into heavy water medium, over the ensuing two months, the lag period became progressively shorter, stabilizing at about 4 days in fully adapted cultures (5% inoculum volume). During this two month period an increase in growth rates also occurred, which was first apparent subsequent to adaptation of the alga to 99.3% D_2O (Figure 3). Culture growth characteristics remained stable once the alga had become fully adapted to D_2O. *S. obliquus* has now been maintained in concentrated heavy water culture for over three years.

Biomass, Lipid and Fatty Acid Yields

S. obliquus was grown in closed batch cultures in both heavy water and normal water Beijerinck's medium to obtain quantitative and qualitative estimates of product yields and to identify the efficacy of selected downstream processing options. Two 5-L culture vessels were used in each experimental trial to provide sufficient algal material for analysis of lipid and fatty acid content and composition.

Pronounced differences in growth rates and biomass yields existed between 5-L H_2O and 5-L D_2O cultures (Figure 5). Newly inoculated 5-L H_2O cultures proceeded to exponential growth phase within 2 days and stationary growth phase (ie, no net increase in biomass) was reached in about 10 days. By contrast, the lag growth phase in 5-L D_2O cultures lasted 3 to 7 days and cultures entered stationary growth phase after approximately 15 days. Growth rates, as measured by light absorbance, were about seven times higher in H_2O medium than in D_2O medium (0.78 and 0.11 O.D./day, respectively). Biomass yields from D_2O cultures, as estimated from absorbance readings at the time of cell harvest, were about one third those obtained from H_2O cultures. However, the mean dry weight of harvested biomass from D_2O cultures was 56% of the mean dry weight values from H_2O cultures (0.84 and 1.5 g D.W./L, respectively; Table 1).

The differences in growth characteristics between algae reared in H_2O and D_2O media were also apparent in the 100 mL maintenance and starter cultures (Figure 6). However, while the growth rate in D_2O medium was comparable in the 100-mL and 5-L cultures, the growth rate for 100-mL H_2O cultures was only half (about 0.38 O.D./day) that obtained in 5-L cultures.

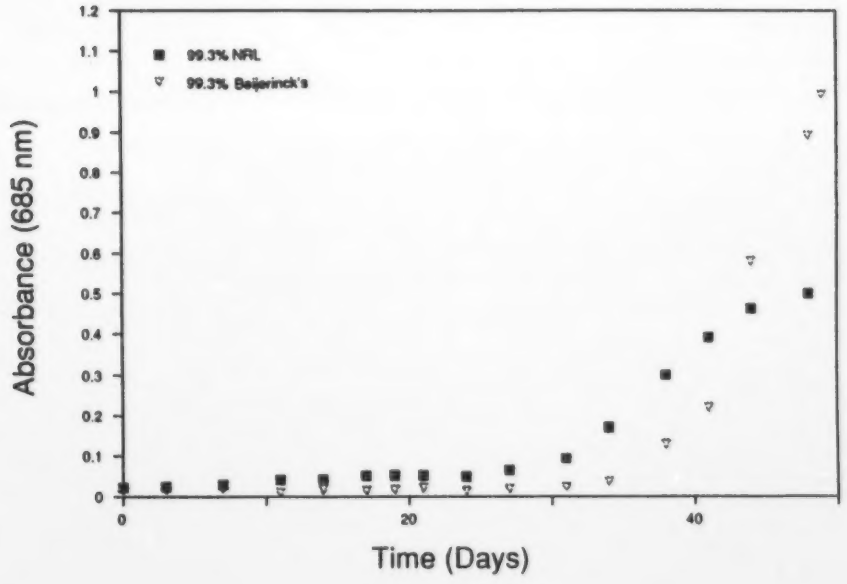

FIGURE 4

GROWTH OF *S. OBLIQUUS* IN NRL - HIGH IRON AND BEIJERINCK'S MEDIA CONTAINING 99.3% D_2O; FOLLOWING INOCULATION FROM PARALLEL CULTURES CONTAINING 97.5% D_2O

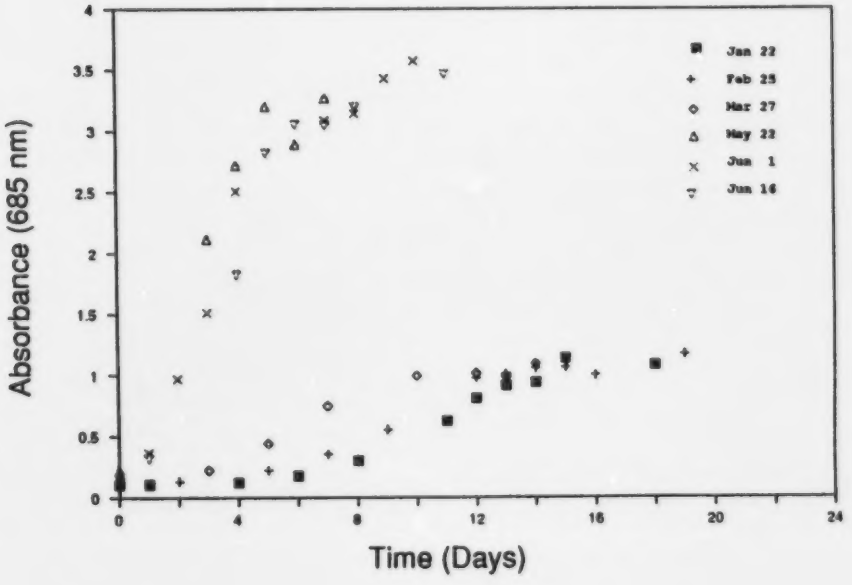

FIGURE 5

GROWTH CURVES FOR *S. OBLIQUUS* IN 5-L D_2O AND 5-L H_2O CULTURES (D_2O MEDIUM: JANUARY 22, FEBRUARY 25, MARCH 27; H_2O MEDIUM: MAY 22, JUNE 1, JUNE 16)

TABLE 1

Biomass Yields for *S. obliquus* Grown in D_2O and H_2O Media; 5-L Cultures

Media	Biomass Yield (g Dry Wt./L) Mean	S.D.	n
D_2O	0.84	0.11	8
H_2O	1.46	0.31	5

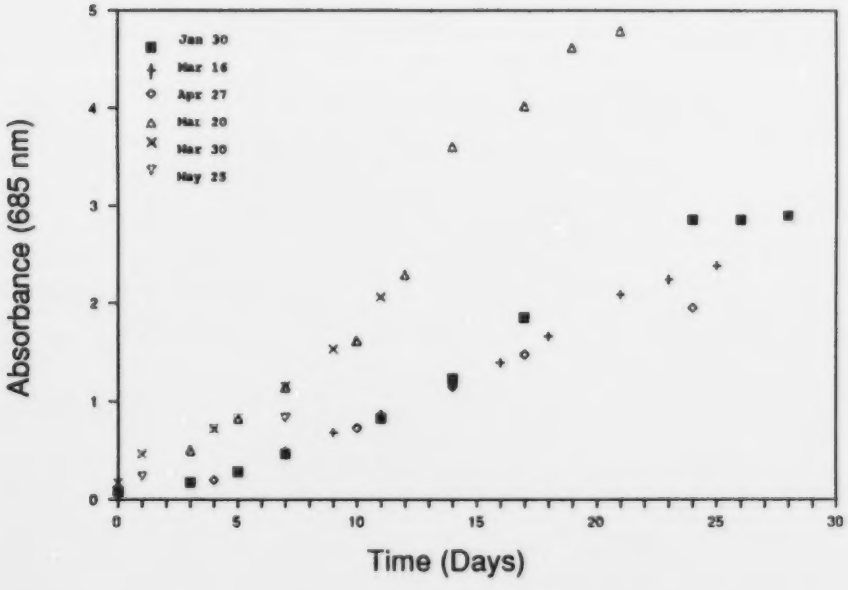

FIGURE 6

GROWTH CURVES FOR *S. OBLIQUUS* IN 100-mL D_2O AND 100-mL H_2O CULTURES (D_2O MEDIUM: JANUARY 30, MARCH 16, APRIL 27; H_2O MEDIUM: MARCH 20, MARCH 30, MAY 25)

It should be noted that the difference in biomass of H_2O and D_2O cultures, estimated by light absorbance or measured as dry weight, suggests that the response between cell biomass and absorbance is curvilinear (ie, decreasing biomass:absorbance ratios at higher optical densities). The relationship between cell biomass and light absorbance was established using dilution curves but only up to absorbance readings of 1.2, which was about one third of the absorbance attained by the 5-L H_2O cultures. Slight curvature (as described above) was noted in two of the calibration curves at the highest cell densities (undiluted samples, O.D. = 1.2). Samples having high cell densities required dilution prior to the measurement of light absorbance and this may have resulted in deviation from expected linearity. The available data did not enable the resolution of the discrepancy in biomass estimated by absorbance versus that measured as dry weight.

Lipid yields for algae grown in H_2O and D_2O media and harvested by centrifugation were comparable, representing about 10% of algal dry weight (Table 2). The higher mean lipid yield (15.8% of cell dry wt.), and greater variability, for algal material harvested by chemical flocculation (CaO) was largely related to the exceptionally high lipid yield (26.6%) obtained in the July 10 trial. However, lipid yields for CaO concentrated cells were consistently higher than for centrifuged material (Appendix 2).

Fatty acid yields were determined for *S. obliquus* grown in H_2O medium (yields were not established for the alga on D_2O medium). Fatty acid yields were compared for algal material harvested by centrifugation or chemical flocculation which was either directly saponified or saponified following methanol-chloroform extraction of lipids (Table 3). Mean yields differed by a factor of two among the four treatments (four independent trials). Higher yields were obtained for cells harvested by centrifugation as opposed to cells harvested by chemical flocculation. Higher fatty acid yields were also obtained when harvested cells were directly saponified as opposed to the treatment involving the extraction of lipids prior to saponification. However, a two-way ANOVA indicated that the differences in fatty acid yields among the four treatments were not significant ($F_{1,12} = 2.73$ for harvest method and 1.01 for recovery method; critical $F = 4.75$, $p<0.05$; Appendix III). The lack of significant differences in fatty acid yields among the four treatment methods can be attributed, in part, to the highly variable results obtained for the centrifugation-direct saponification treatment and to the limited number of trials that were conducted.

The fatty acid content of the algal material, which represents the yield of desired product, was approximately 3 to 5% of cell dry weight.

TABLE 2

Lipid Yields for *S. obliquus* Grown in D_2O and H_2O Media; 5-L Cultures

Media	Harvest Method	Lipid Yield (% of Dry Wt.) Mean	S.D.	n
D_2O	Cent	10.1	2.72	7
H_2O	Cent	9.7	3.04	4
H_2O	CaO	15.8	7.58	4

TABLE 3

Fatty Acid Yields For *S. obliquus* Grown in 5-L H_2O Culture as Influenced by Two Harvesting and Two Recovery Methods

Harvest Method	Recovery Method	Fatty Acid Yield (g/L) Mean	S.D.	n	(% of Dry Wt.) Mean	S.D.	n
Cent	Dir Sap	0.071	0.047	4	5.13	2.73	4
Cent	M/C-Sap	0.062	0.019	4	4.28	0.75	4
CaO	Dir Sap	0.053	0.012	4	3.88	0.78	4
CaO	M/C-Sap	0.036	0.009	4	2.85	1.48	4

Fatty Acid Composition

The predominant fatty acids from algae grown in H_2O medium, and the only identified fatty acids from cells grown in D_2O medium, were palmitic, oleic and 11,14-eicosadienoic acid (Table 4). The proportion of palmitic acid was consistently high in samples from both medium and tended to be slightly higher in D_2O samples compared to H_2O samples (means of 59% and 48%, respectively). A high proportion of oleic acid was consistently present in H_2O material but it's abundance in D_2O material was highly variable (means of 32% and 24% respectively). The relative abundance of 11,14-eicosadienoic acid was highly variable in both sets of samples. An additional six fatty acids were identified from cells grown in H_2O medium. Stearic acid was a component of all H_2O material, accounting for approximately 5.5% of the total lipid content.

There was little overall difference in the proportion of saturated to unsaturated fatty acids between the H_2O and D_2O materials. Qualitative examination of the mass spectra for the deuterated acids indicated that the molecules were more than 95% deuterated.

Copper Tolerance

S. obliquus tolerated a wide range in copper concentrations (Figure 7). Good growth was obtained between copper concentrations of 0.22 and 18.3 mg/L while no growth occurred at concentrations of 36.6 mg/L and higher. Maximum growth was obtained at a copper concentration four times higher (1.53 mg/L) than that used in standard Beijerinck's medium (0.39 mg/L).

DISCUSSION

The goals of the current study were to establish growth of *Scenedesmus obliquus* in concentrated heavy water medium, develop procedures for the recovery of fatty acid end products from harvested biomass and provide a preliminary evaluation of biosynthesis as a means of producing perdeuterated organic compounds.

The prolonged period (3 months) required to adapt *S. obliquus* to concentrated heavy water (>99.6%) medium has been noted previously for this and other green algae species (Crespi et al. 1959, Flaumenhaft et al. 1960, Katz and Crespi 1966). A gradual reduction in growth rates and biomass yields occurred as D_2O concentration increased up to 93.4% while the lag growth period remained largely unchanged. This suggests that the alga continued to use normal water for biosynthesis although the H_2O concentration decreased with each successive transfer. Adaptation to heavy water was apparently forced as D_2O concentration increased from 93.4% to 99.3% as indicated by the prolonged lag growth period and reduced

TABLE 4

Fatty Acids Derived From *Scenedesmus obliquus* Grown in H_2O and D_2O Media; 5-L Cultures

Systematic Name	Common Name	Carbon Atoms	Structure	Percent Fatty Acid Composition (Mean and Range)* H2O Culture	D2O Culture
Nonanedioic Acid (T)	Azelaic Acid	9	COOH(CH2)7COOH	0.2 (0-0.6)	0
10-Undecenoic Acid (T)	Declid Acid	11	CH2=CH(CH2)8COOH	0.6 (0-1.9)	0
Tetradecanoic Acid	Myristic Acid	14	CH3(CH2)12COOH	0.2 (0-0.5)	0
Cis-9-Hexadecenoic Acid	Palmitoleic Acid	16	CH3(CH2)5CH=CH(CH2)7COOH	0.3 (0-1.5)	0
Hexadecanoic Acid	Palmitic Acid	16	CH3(CH2)14COOH	47.6 (30.9-63.5)	58.9 (38.5-74.8)
Cis,cis-9,12-Octadecadienoic Acid	Linoleic Acid	18	CH3(CH2)4CH=CHCH2CH=CH(CH2)7COOH	0.3 (0-1.7)	0
Cis-9-Octadecenoic Acid	Oleic Acid	18	CH3(CH2)7CH=CH(CH2)7COOH	31.9 (19.7-59.0)	24.0 (3.7-61.6)
Octadecanoic Acid	Stearic Acid	18	CH3(CH2)16COOH	5.5 (4.3-7.5)	0
Cis,cis-11,14-Eicosadienoic Acid (T)		20	CH3(CH2)4CH=CHCH2CH=CH(CH2)9COOH	13.3 (0-29.2)	17.1 (0-29.8)

* Analysis of 5 H2O and 3 D2O Cultures

T Tentative Identification

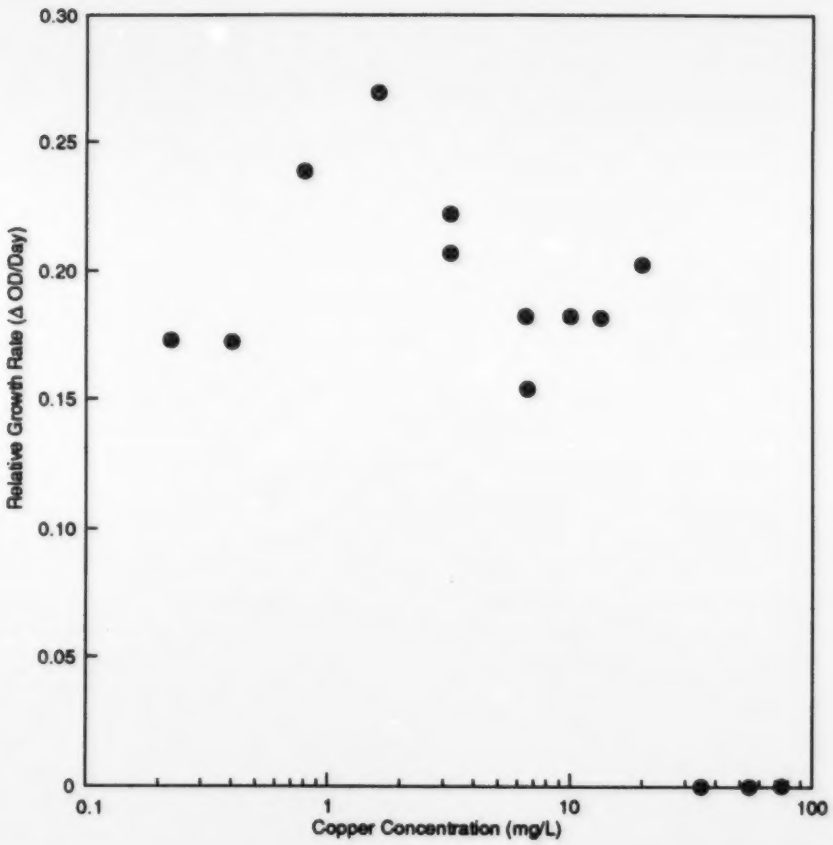

FIGURE 7

RELATIVE GROWTH RATES FOR CULTURES OF S. OBLIQUUS POISED AT DIFFERENT COPPER CONCENTRATIONS

growth rates. It has been demonstrated, however, that the prolonged adaptation process may not be universal, with one alga, *Neochloris oleoabundans*, found to have a propensity for growth in heavy water (Kyle et al. 1987). Once fully adapted, growth of green algae can be maintained in heavy water for a number of years as demonstrated in this and other studies (Flaumenhaft et al. 1960, Katz and Crespi 1966).

Growth rates for algae that are fully adapted to heavy water are typically lower than growth rates in H_2O medium (Crespi et al. 1959, Katz and Crespi 1966, Kyle et al. 1987). In this study, biomass yields for *S. obliquus* grown on D_2O medium were also lower (56%) than yields obtained from H_2O culture. However, others have reported comparable yields from either H_2O or D_2O cultures suggesting that the ultimate limit on biomass yield is imposed by light availability (Katz and Crespi 1966, Taecker et al. 1971).

The 5-L culture vessels used in our experiments provided for good algal growth but were not optimized for biomass production and it can be assumed that growth was light limited. Our biomass yield of 0.84 g/L was low compared to yields of up to 8 g dry wt/L reported for heavy water cultures of *S. obliquus* under culture configurations which allow for ample light supply (Crespi et al. 1959). It was previously estimated that lipid yields approaching 3 g/L could be obtained under batch D_2O culture conditions given a cell lipid content of 40% of cell dry weight (Dunstall 1985). Biomass yields of up to 12 g dry wt./L have been reported for the green alga, *N. oleoabundans*, which was accompanied by an increase (to 40% of biomass dry weight) in stable extractable lipids in heavy water culture (Kyle et al. 1987).

The maximum obtainable lipid yield is a function of algal growth rate (biomass production) and cell lipid content. Little lipid accumulation occurs when conditions favour cell proliferation. High cell lipid content is achieved at the expense of biomass production (Ratledge 1982). Culture conditions therefore need to be controlled to maximize biomass production and subsequently manipulated to enhance lipid production. The latter is accomplished by stressing the cells, with the most common approach being to limit nitrogen availability. Severe nitrogen limitation results in a two to three fold increase in lipid content, mostly triacylglercerols (Richardson et al. 1969, Shifrin and Chisholm 1980, Shifrin 1984, Niehof et al. 1987, Suen et al. 1987). An approximate inverse relationship exists between cell nitrogen content and cell lipid content. Maximum cell lipid levels are attained within 4 to 9 days following onset of nitrogen starvation (Shifrin and Chisholm 1981).

Lipid yields that were 10% of biomass dry weight were obtained for *S. obliquus* in the current study, for cultures that were harvested immediately following exponential growth phase. A similar lipid yield for actively growing cultures of this species was reported by

Aaronson et al. (1980). An increase in lipid content to 60% of cell dry weight was obtained for the same strain of *S. obliquus* as used in our study, by limiting the available nitrogen, increasing iron concentration in the medium and prolonging the incubation period (Niehof et al. 1987).

In this study, *S. obliquus* was found to tolerate relatively high concentrations of copper (up to 18.3 mg/L), suggesting that the opportunity exists for enhancing lipid production by screening for copper tolerant strains. Copper tolerant clones from several algal species have been found to produce more lipid in response to nitrogen deficiency than copper intolerant clones.

The dominant fatty acids from *S. obliquus* were palmitic (16:0) and oleic (18:1) as found in this study and in studies conducted at the U.S. Naval Research Laboratory (Niehof et al. 1986, 1987, Ross et al. 1986). In both cases, a higher relative proportion of palmitic acid and a lower proportion of oleic acid was present in algae grown in D_2O medium compared to algae grown in H_2O medium. The prominence of these two fatty acids in *S. obliquus*, and the considerable influence of culture conditions such as temperature, light quality and quantity and nutrient availability on cellular fatty acids composition has been described in a number of additional studies (Aaronson et al. 1980, Materassi et al. 1980, Pohl et al. 1971, Shifrin and Chisholm 1980, Shifrin 1984).

Other fatty acids identified by NRL for their heavy water cultures of *S. obliquus*, included 16:1, 18:0, 18:2 and 18:3 (Ross et al 1986, Niehof et al. 1986, 1987). Linoleic acid (18:2) was the major lipid identified by Graff et al. (1970) in their D_2O cultures of *S. obliquus*, although all saturated and unsaturated 16 and 18 carbon fatty acids were present. Several of these fatty acids were present in H_2O-grown algae in our study but not from D_2O-grown cells. Cis,cis-11,14-eicosadienoic acid (20:2) was the third most abundant fatty acid in our D_2O and H_2O samples which was somewhat surprising since the relative abundance of C_{14} and C_{20} fatty acids tend to be low in green algae (Shifrin 1984; Shifrin and Chisholm 1980). However, the 20:2 fatty acid was obtained as a tentative identification.

Once the organic compounds have been deuterated limited exchange of hydrogen with deuterium occurs during product recovery. Fatty acids recovered in this study were more than 95% deuterated and Ross et al. (1986) found the average atom-percent deuteration to be 98.3%. Graff et al. (1970) found H-D exchange to be about 3%, which was attributed to the hydrocarbon solvents used to purify the lipid material.

A major constraint to the mass cultivation of microalgae for the biosynthesis of deuterated materials is the economy of harvesting small algal cells dispersed within large volumes of medium and in subsequent recovery of desired products. However, the economics of

these processes is related to product value (Ratledge 1982). A method involving the use of calcium oxide to facilitate chemical flocculation and subsequent settling of algal cells was adapted for separating algae from culture medium. The use of CaO, rather than CaOH, eliminated the possibility of contaminating recovered products through H-D exchange.

Fatty acid yields were compared for algal material harvested by centrifugation or chemical flocculation which was either directly saponified or saponified following methanol-chloroform extraction of lipids. The use of the flocculation-settling method, followed by direct saponification of the concentrated cell material, was an effective and promising alternative to centrifugation, lipid extraction and saponification for the recovery of fatty acid products. However, comparison of the four treatments involved only a limited number of trials (4) and additional testing is recommended prior to implementation. Further downstream processing of recovered fatty acids, including breaking of 16 and 18 carbon fatty acids into lower molecular weight alkanoic acids and subsequent synthesis of the polyolesters used in lubricant applications has been described by Kyle et al. (1987).

Algae grown photosynthetically are capable of producing a wide range of perdeuterated organic compounds and uses for these materials may have a number of applications (Robins 1984, Ross et al. 1987). Biological substitution is expected to proceed differently than chemical substitution, with every H-position having an equal chance of being replaced by a deuterium atom. Hydrogen-deuterium exchange during chemical substitution likely proceeds from the weakest to the strongest bonds and a high degree of deuteration is required to increase stability of the whole molecule (Schindler and Wolock 1982). In addition to ease of production and a high atom percent deuteration, other advantages of biosynthesis over chemical substitution include the ability to deuterate organic compounds that are difficult to deuterate chemically (ie, labile compounds) and in greater purity (Tulloch 1979, Ross et al. 1987, Niehof et al. 1986). Cost effective means for biosynthesizing deuterated organic compounds would necessitate the recycling and periodic upgrading (distillation) of spent culture media (eg, Lazareva et al. 1974).

Post-Script: Our D_2O-adapted culture of *Scenedesmus obliquus* was transferred to a University of Guelph research team for studies on deuterium applications.

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APPENDIX I

Growth Media

	NRL[1] High Iron (g/L)	NRL[1] Low Iron (g/L)	Beijerinck's[2] Medium (g/L)
$MgSO_4.7H_2O$	0.49	0.49	0.02
NH_4NO_3	0.20	0.20	0.15
Na_2HPO_4	0.10	0.10	
K_2HPO_4			0.72
KH_2PO_4			0.36
$KHCO_3$	0.10	0.10	
$Ca(NO_3)_2.4H_2O$	0.22	0.22	
NaCl	0.010	0.010	
$CaCl_2.2H_2O$			0.010
$FeSO_4.7H_2O$	0.014	0.005	0.005
Micronutrients:			
H_3BO_4	0.010	0.010	0.010
$CuSO_4.5H_2O$	0.0015	0.0015	0.0015
EDTA	0.050	0.050	0.050
$ZnSO_4.7H_2O$	0.022	0.022	0.022
$MnCl_2.4H_2O$	0.0050	0.0050	0.0050
$CoCl_2.6H_2O$	0.0015	0.0015	0.0015
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0010	0.0010	0.0010

[1] Modified NRL medium (Neihof et al. 1986) with substitution of Beijerinck's micronutrient solution.

[2] Beijerinck's medium (Nichols 1975).

Appendix II

Biomass, Lipid and Fatty Acid Yields From *Scenedesmus obliquus* Grown in 5-L D_2O and 5-L H_2O Cultures

Date 1987	Medium Water	Culture Flask	Sample #	Sample Vol. L	Biomass Yield g D.W./L	Harvest Method	Extraction Method*	Lipid Yield g/L	Fatty Acid Yield g/L	M/C Lipid as % of D.W.	F.A. as % of D.W.
Jan 22	D2O	5 L	1	4.88	0.83	Cent	M/C-a	0.0585		7.1	
	D2O	5 L	2	4.83	0.79	Cent	M/C-a	0.0834		10.6	
Feb 25	D2O	5 L	1	4.82	0.94	Cent	M/C-a	0.0657		7.0	
(A)	D2O	5 L	2	4.84	0.85	Cent	M/C-a				
Mar 27	D2O	5 L	1	4.96	0.79	Cent	M/C	0.0649		8.2	
(B)	D2O	5 L	2	4.73	0.89	Cent	M/C	0.1060		11.9	
Apr 10	H2O	2 X 5 L	1	2.44	0.95	Cent	Dir Sap		0.0577		6.1
(C)		Combined	2	2.44	(combined)	Cent	M/C - Sap	0.0537	0.0427	5.7	4.5
		div by 4	3	2.44		CaO	Dir Sap		0.0423		4.4
			4	2.44		CaO	M/C - Sap	0.1126	0.0472	11.9	5.0
Apr 28	D2O	5 L	1	4.72	0.63	Cent	M/C	0.0801		12.7	
(D)		5 L	2	4.86	0.91	Cent	M/C	0.1246		13.7	
May 22	H2O	2 X 5 L	1	2.40	1.41	Cent	Dir Sap		0.0294		2.1
		Combined	2	2.40	(combined)	Cent	M/C - Sap	0.1274	0.0472	9.0	3.3
		div by 4	3	2.40		CaO	Dir Sap		0.0653		4.6
			4	2.40		CaO	M/C - Sap	0.1346	0.0347	9.5	2.5
Jun 1	H2O	2 X 5 L	1	2.40	1.52	Cent	M/C - Sap	0.1887	0.0782	12.4	5.1
		Combined	2	2.40	1.51	Cent	Dir Sap		0.0591		3.9
		div by 4	3	2.40	1.52	CaO	M/C - Sap	0.2276	0.0248	15.0	1 6
			4	2.40	1.54	CaO	Dir Sap		0.0440		2.9
Jun 18	H2O	2 X 5 L	1	1.50	1.72	Cent	M/C - Sap	0.2173	0.0611	12.6	3.6
(E)		Combined	2	1.50	2.04	Cent	M/C - Sap	0.2135	0.1019	10.5	5.0
		div by 6	3	1.50	1.78	Cent	M/C - Sap	0.2124	0.0704	11.9	4.0
			4	1.50	1.72	CaO	Dir Sap		0.0487		2.8
			5	1.50	1.70	CaO	Dir Sap		0.0670		3.9
			6	1.50	1.67	CaO	Dir Sap		0.0678		4.1
Jul 10	H2O	2 X 5 L	1	1.50	1.66	Cent	Dir Sap		0.1501		9.0
		Combined	2	1.47	1.63	Cent	Dir Sap		0.1239		7.6
		div by 6	3	1.50	1.64	Cent	Dir Sap		0.1397		8.5
			4	1.50	1.66	CaO	M/C - Sap	0.3621	0.0322	21.8	1.9
			5	1.50	1.58	CaO	M/C - Sap	0.3103	0.0381	19.6	2.4
			6	1.50	1.58	CaO	M/C - Sap	0.6081	0.0407	38.5	2.6

* M/C-a - Double extraction: Methanol:Chloroform:Cell Suspension, 2:1:0.8

M/C - Double extraction: Methanol:Chloroform:Cell Suspension, 2:2:1.8; Second extraction acidified

Dir Sap - Direct Saponification (see methods)

For June 18 and July 10 experiments, cell debris and CaO was removed before adjusting solvent pH to 7

Appendix III

Fatty Acid Yields from 5-L H_2O Cultures of Scenedesmus obliquus: Comparison of Two Harvesting Methods and Two Fatty Acid Recovery Methods (2-Way ANOVA)

Fatty Acid Yield (g/L)

		A: Direct	A: Saponification	A: MeOH/Chloroform - Saponification	
B	Cent	0.058	0.029	0.043	0.047
		0.059	0.138	0.078	0.078
	CaO	0.042	0.065	0.047	0.035
		0.044	0.061	0.025	0.037

B \ A	1	2
	3	4

2-Way ANOVA

Source	Sum Squares	df	Mean Square	F-Ratio
Treat A	7.0225E-4	1	7.0225E-4	1.013226
Treat B	1.89225E-3	1	1.89225E-3	2.730191
AXB	5.624999E-5	1	5.624999E-5	8.115906E-2
Error	8.317E-3	12	6.930833E-4	
Total	1.096775E-2	15		

Fatty Acid as % of Dry Weight

		B: Direct	B: Saponification	B: MeOH/Chloroform - Saponification	
B	Cent	6.1	2.1	4.5	3.3
		3.9	8.4	5.1	4.2
	CaO	4.4	4.6	5.0	2.5
		2.9	3.6	1.6	2.3

B \ A	1	2
	3	4

1 = Apr 10
2 = May 22
3 = Jun 1
4 = Jun 18 + Jul 10

2-WAY ANOVA

Source	Sum Squares	df	Mean Square	F-Ratio
Treat A	3.515625	1	3.515625	1.299977
Treat B	7.155625	1	7.155625	2.645944
AXB	3.06251E-2	1	3.06251E-2	1.132428E-2
Error	32.4525	12	2.704375	
Total	43.15437	15		

Critical $F_{(1,12)} = 4.75$; $p \leq 0.05$